

**ZINC-REGULATED PROKARYOTIC EXPRESSION CASSETTES**

5 The invention relates to the production of heterologous proteins in gram-positive bacteria, in particular lactic acid bacteria.

Besides their conventional uses in the agro-foods industry, lactic acid bacteria are currently increasingly used as host cells for the production of heterologous proteins of interest. These proteins of  
10 interest can be very varied in nature, and it is therefore desirable to have as large a choice as possible of expression tools in order to be able to optimize the production thereof as a function of the specificities of each one of them.

15 In general, it is necessary to use strong promoters that make it possible to obtain a sufficient level of expression of the gene of interest. In certain cases, constitutive promoters can be used; in other cases (for example when the product of the gene of  
20 interest is toxic for the host bacterium or there is a risk of it interfering with the metabolism thereof), it is preferable to use inducible promoters that make it possible to initiate or to stop the expression at the desired time.

25 Although lactic acid bacteria have many genes whose transcription is regulated by various factors, at the current time, there is only a relatively restricted choice of inducible promoters that can be used in practice for constructing expression cassettes for  
30 genes of interest (for review, cf. DE VOS, Curr. Op. Microbiol., 2, 289-295, 1999). In fact, this use requires not only that the promoters concerned can be regulated, but also that there exists a sufficient expression differential between the various induction  
35 states; ideally, the expression should reach a high level under induction conditions and should be able to be completely blocked under non-induction conditions. In addition, it is necessary to be able to readily

control the factors involved in the regulation of these promoters.

In previous studies, aimed at identifying exported proteins from *L. lactis* (POQUET et al., J. Bacteriol., 180, 1904-1912, 1998), the inventors cloned, by fusion with the reporter gene  $\Delta_{spNuc}$ , a fragment of genomic DNA of the *L. lactis* strain MG1363, comprising a gene called, at the time, *nlp3* (New LipoProtein 3), the product of which exhibits homologies with an *S. pneumoniae* protein involved in the transport of metals. The sequence of this fragment is available on GENBANK under the number U95834.

The inventors also observed that the *nlp3* gene was negatively regulated by divalent metal cations, in particular  $Zn^{2+}$  (POQUET et al. "Use of a new reporter tool to demonstrate metal regulation of *nlp3*, a gene putatively involved in metal uptake in *Lactococcus lactis*"; 6th Symposium on Lactic Acid Bacteria, Veldhoven, The Netherlands, September 19-23, 1999).

Furthermore, in the context of the complete sequencing of the *L. lactis* IL1403 genome, the *nlp3* gene, renamed *zitS*, was identified as a constituent of an operon, called *zitRSQP* (BOLOTIN et al., Antonie van Leeuwenhoek, 76, 27-76, 1999; BOLOTIN et al., Genome Res. 11, 731, 2001). By homology with known sequences, putative functions in zinc transport were attributed to the genes of this operon. Thus, the product of the *zitP* gene is thought to constitute the permease of the transport system, the products of the *zitS* gene and of the *zitQ* gene are thought to ensure, respectively, binding with the substrate and binding with ATP, and the product of the *zitR* gene, which exhibits homologies with the *marR* transcriptional repressor family, is thought to be involved in the regulation of zinc transport.

Up until this point, use of the *zitRSQP* operon regulatory system for controlling the expression of heterologous genes had not been envisioned. In fact, although a negative regulation may be initiated by the

addition of zinc (POQUET et al., 1999, mentioned above), the basal level of expression observed in the absence of this negative regulation did not appear to be sufficient to allow satisfactory production of proteins of interest. In addition, it was not known whether the putative repressor ZitR was effectively involved in repression of the expression of this operon, or whether other regulators, in particular the pleiotropic *flp* regulators, described as being involved in the regulation of zinc transport in *L. lactis* (GOSTICK et al., Mol. Microbiol., 31, 1523-35, 1999; SCOTT et al., FEMS Microbiol. Lett., 192, 85-89, 2000), could also be involved, either as corepressors or, conversely, as possible activators.

Now, in continuing their studies on the regulation of the *zitRSQP* operon, the inventors noted, in the presence of very low concentrations of  $Zn^{2+}$ , a maximum level of expression that was much greater than could be assumed according to prior experiments, and which made it possible to attain an induction factor of greater than 100. In addition, they noted that the expression was independent of *flp* regulators, and could be entirely controlled by means of the ZitR protein.

Studying the structure of the *L. lactis zitRSQP* operon promoter has allowed the inventors to demonstrate, besides the elements conventionally present in bacterial promoters, namely the -35 (TTGACA) and -10 (TATAAT) boxes separated by 17 bp, a palindromic sequence overlapping the -35 box, which very probably represents the ZitR-binding site.

The observations reported above make it possible to assume that *zitRSQP* is regulated according to the following mechanism: the ZitR repressor can form, with intracellular  $Zn^{2+}$ , a complex exhibiting a very substantial affinity for the binding site overlapping the -35 box; the ZitR- $Zn^{2+}$  complex bound to the palindrome prevents access of the RNA polymerase to the -35 box, and therefore represses the transcription; on the other hand, the non-complexed form of ZitR does not

bind to the -35 site, allowing transcription of the operon, which is then carried out with great efficiency.

Regulation of the *zitRSQP* operon by means of ZitR therefore depends on the intracellular concentration of  $Zn^{2+}$ , which itself depends on the  
5 availability of  $Zn^{2+}$  in the culture medium.

The *zitRSQP* operon very probably represents a very high affinity zinc transport system that is only used by the bacterium under very severe conditions of zinc deficiency, so as to allow cell survival; on the  
10 other hand, under the usual conditions of culturing on rich media, the zinc is abundantly present in the environment and is transported into the cell by systems with a lower affinity than the ZitSPQ complex, the  
15 synthesis of which is then repressed.

These properties of the *L. lactis zitRSQP* operon regulatory system demonstrated by the inventors make it possible to propose its use for the production of proteins of interest in host bacteria, especially  
20 gram-positive bacteria, and in particular lactic acid bacteria.

A subject of the present invention is the various aspects of this use.

According to a first variant, a subject of the present invention is an expression cassette consisting  
25 of:

- a bacterial promoter, hereinafter called  $p_{zn}$ , containing a binding site for the *Lactococcus lactis* ZitR protein, which site comprises the following  
30 sequence:

AAAATAANGTNNNNNNNTTGACATTATTTT

(SEQ ID NO:1),

in which TTGACA represents the -35 box of said promoter, and N represents A, C, G or T;

- 35 - a sequence encoding a polypeptide exhibiting at least 80%, preferably at least 85%, and entirely preferably at least 95% identity with the *Lactococcus lactis* ZitR protein (GENBANK AAK06214), placed under the transcriptional control of said promoter;

- at least one restriction site allowing the insertion of a nucleotide sequence of interest under the transcriptional control of said promoter.

According to a preferred embodiment of the present invention, the  $p_{zn}$  promoter comprises the following sequence:

AAAAATAANGTNNNNNNNTTGACATTATTTTTNNNNNNNNNTATAAT

(SEQ ID NO:2)

in which TATAAT represents the -10 box of said promoter.

According to another preferred embodiment of the present invention, said  $p_{zn}$  promoter contains a binding site for the *Lactococcus lactis* ZitR protein comprising the following sequence:

AAAAATAAYGTAACTGGTTGACATTATTTTT

(SEQ ID NO:3),

in which Y represents T or C.

By way of example of  $p_{zn}$  promoters that can be used for constructing an expression cassette in accordance with the invention, mention will be made of:

- the  $p_{zn}$  promoter of the *Lactococcus lactis* strain MG1363, which comprises the sequence:

AAAAATAATGTAACTGGTTGACATTATTTTTACTTTGCTATATAATTAACCGTA

(SEQ ID NO:4);

- the  $p_{zn}$  promoter of the *Lactococcus lactis* strain IL1403, which comprises the sequence:

AAAAAATAACGTAACTGGTTGACATTATTTTTCTTTGCTATATAATTAACCGTA

(SEQ ID NO:5).

According to a second variant, a subject of the present invention is an expression cassette consisting of:

- a bacterial promoter  $p_{zn}$ , as defined above;  
- at least one restriction site allowing the insertion of a nucleotide sequence of interest under the transcriptional control of said promoter.

A subject of the present invention is also expression cassettes resulting from the insertion of a nucleotide sequence of interest into an expression cassette in accordance with the first or with the

second variant of the invention, under the transcriptional control of the  $p_{zn}$  promoter.

Said nucleotide sequence of interest may be any sequence that it is desired to express under the transcriptional control of the  $p_{zn}$  promoter. It may in particular be any sequence encoding a heterologous protein of interest that it is desired to produce in a host bacterium; said protein may, where appropriate, be a fusion protein, combining polypeptide sequences of diverse origin.

However, the expression cassettes in accordance with the first variant of the invention and comprising all or part of the sequence encoding the *L. lactis* ZitS protein, fused to a reporter gene, are excluded.

Expression cassettes in accordance with the invention may, where appropriate, also comprise the elements required for targeting the protein of interest to the cell surface, or for its secretion into the culture medium.

In this context, a subject of the present invention is expression cassettes resulting from the insertion of a nucleotide sequence encoding an extracellular targeting peptide, and of at least one restriction site allowing the cloning of a nucleotide sequence of interest as a translational fusion with said targeting peptide, under the transcriptional control of the  $p_{zn}$  promoter, into an expression cassette in accordance with the invention.

Said targeting peptide may, for example, be a secretion signal peptide, a transmembrane domain, a signal for anchoring to the wall, etc.

Many targeting peptides that can be used in the context of the present invention are known in themselves. By way of nonlimiting examples, mention will be made of the peptides described in the publication by POQUET et al. (1998, mentioned above), or in the publication by LE LOIR et al. (Appl. Environ. Microbiol., 67, 4119-4127, 2001).

For the production of secreted proteins, a preferred extracellular targeting peptide is the signal peptide of the *L. lactis* Exp4 protein, which corresponds to the sequence:

5 MKKINLALLTLATLMGVSSSTAVVFA (SEQ ID NO: 6).

A subject of the present invention is also any recombinant vector comprising an insert consisting of an expression cassette in accordance with the invention.

10 A subject of the present invention is also gram-positive bacteria transformed with at least one expression cassette in accordance with the invention.

Preferably, they are lactic acid bacteria, in particular lactococci, lactobacilli or streptococci  
15 that are thermophilic.

Where appropriate, they may be bacteria originating from bacterial strains comprising one or more modifications of their genome, aimed at improving the production and/or the secretion of proteins expressed in said bacteria, and/or at preventing their  
20 degradation. For example, in the context of the production of exported proteins, use may be made of a bacterial strain in which the PrtP protease activity and/or the HtrA protease activity are inactive, such as  
25 that described in PCT application WO 00/39309, or a bacterial strain that overproduces a protein that makes it possible to stabilize the exported proteins, such as the *Lactococcus lactis* Nlp4 protein or one of its homologs (POQUET et al. 1998, publication mentioned  
30 above).

A subject of the present invention is also the use of expression cassettes or of recombinant vectors in accordance with the invention, for producing proteins of interest in a gram-positive bacterium, in  
35 particular lactic acid bacteria.

The expression cassettes in accordance with the first variant of the invention can be used in a host bacterium, for controlling the time at which a gene of

interest inserted into the cloning site is expressed, and the level of this expression.

When the host bacterium is cultured in the presence of an amount of zinc that is in excess with respect to its needs, the expression of the gene of interest is completely repressed. Depletion of  $Zn^{2+}$  in the culture medium, which can be carried out simply by adding a divalent cation-chelating agent such as EDTA makes it possible to lift the repression, and to bring about expression of the gene. The level of expression can be readily regulated through the amount of chelating agent added.

The host bacterium can also be cultured in a medium comprising an amount of zinc that is just sufficient to cover its needs during a given period of the culture (for example during the growth phase). In this case, the depletion of zinc at the end of this period brings about expression of the gene of interest.

In this context, a subject of the present invention is a method for producing a protein of interest in a gram-positive bacterium, and in particular a lactic acid bacterium, characterized in that it comprises:

- introducing into said bacterium an expression cassette in accordance with the first variant of the invention, comprising a sequence encoding said protein of interest;

- culturing said bacterium in a medium containing an amount of  $Zn^{2+}$  that is sufficient to repress the expression of said protein;

- inducing the production of said protein by  $Zn^{2+}$  depletion of said medium;

- recovering the protein produced.

According to a preferred embodiment of the method in accordance with the invention, the  $Zn^{2+}$  depletion of said medium is obtained by adding a divalent cation-chelating agent.

According to another preferred embodiment of the present invention, the  $Zn^{2+}$  depletion of said medium



is obtained by culturing the bacterium until depletion of the  $\text{Zn}^{2+}$  initially present in the medium.

According to the experiments carried out by the inventors on the *L. lactis* model strain MG1363, an amount of  $\text{Zn}^{2+}$  that is sufficient to repress the expression under the control of the  $p_{\text{zn}}$ /ZitR system can be maintained throughout the duration of the culturing by using a medium containing, at the beginning of culturing, of the order of 1 to 2  $\mu\text{M}$  of  $\text{Zn}^{2+}$ , it being possible for the total repression threshold to be estimated at between 100 nM and 1  $\mu\text{M}$  of  $\text{Zn}^{2+}$ . The  $\text{Zn}^{2+}$  concentration in the medium below which complete lifting of the repression of  $p_{\text{zn}}$  is obtained is very low (of nanomolar order, and at most a few nanomolar). The amount of divalent cation-chelating agent required in order to effect the  $\text{Zn}^{2+}$  depletion and induce the expression under the control of the  $p_{\text{zn}}$  promoter varies according to the amount of  $\text{Zn}^{2+}$  initially introduced into the culture medium; by way of indication, in the case of the MG1363 strain, for a rich culture medium such as M17 medium, a  $\text{Zn}^{2+}$  depletion making it possible to induce maximum expression can be obtained using an EDTA concentration of the order of 0.1 mM; in SA medium, which contains 10 nM of  $\text{Zn}^{2+}$ , a  $\text{Zn}^{2+}$  depletion making it possible to induce maximum expression can be obtained using an EDTA concentration of the order of 0.01 mM.

The amounts of  $\text{Zn}^{2+}$  and of cation-chelating agent mentioned above are given by way of indication. Based on these indications, and on the other information provided by the description of the present invention, those skilled in the art can readily determine, by means of prior tests carried out, for example, by placing a reporter gene under the control of the  $p_{\text{zn}}$ /ZitR system in an expression cassette in accordance with the invention, the most suitable amounts according to the bacterial species or strain concerned, the operating conditions used, such as the medium used, the methods of adding  $\text{Zn}^{2+}$  and/or chelating

agent (for example, all at once, in several steps, continuously, etc.), and the desired level of expression.

5 Expression cassettes in accordance with the second variant of the invention will preferably be used in strains of bacteria, in particular of lactococci, in which the endogenous ZitR repressor is inactive, along with, optionally, the ZitSPQ complex. Under these conditions, the  $p_{zn}$  promoter constitutes a strong  
10 promoter, allowing expression of the protein of interest throughout the duration of the culture. The inactivation of the ZitR receptor and of the ZitSPQ complex can be carried out in a manner known in itself, in particular by site-directed mutagenesis of the  
15 *zitRSQP* operon.

In this context, a subject of the present invention is a method for producing a protein of interest in a gram-positive bacterium in which the endogenous ZitR repressor is inactive, characterized in  
20 that it comprises:

- introducing into said bacterium an expression cassette in accordance with the second variant of the invention, comprising a sequence encoding said protein of interest;
- 25 - culturing said bacterium;
- recovering the protein produced.

The present invention can be implemented, for example:

- in the field of the production of  
30 heterologous proteins of therapeutic interest by genetic engineering, in order to have better control of the production of these proteins by the cultures of transformed bacteria;

- in the agrofoods industry, in particular in  
35 the production of fermented products, for controlling, according to the fermentation stage, the production of proteins of interest that make it possible in particular to influence the quality of the finished fermented product.

The present invention will be understood more thoroughly from the additional description which follows, which refers to nonlimiting examples illustrating the construction of expression cassettes in accordance with the invention.

**EXAMPLE 1: REGULATION OF *ZIT* BY  $Zn^{2+}$  IN *L. LACTIS***

The expression of *zit* as a function of the concentration of  $Zn^{2+}$  in the medium is measured by means of 2 different techniques:

\* Measurement of the Nuc activity of the fusion ZitRS- $\Delta_{sp}$ Nuc, carried by the plasmid pVE8020, in the *L. lactis* strain MG1363. The plasmid pVE8020 results from the cloning of the fragment of chromosomal DNA from the MG1363 strain corresponding to  $p_{zn}zitRzitS'$  (GenBank U95834) in the plasmid pFUN (POQUET et al., 1998, mentioned above; GenBank AF038666).

\* Quantification of the *zitS* mRNA in the wild-type MG1363 strain (endogenous expression of *zitS*) and in the MG1363 strain transformed with pVE8020.

**Effect of zinc on the Nuc activity under the control of the  $p_{zn}$  promoter**

Two types of experiments were carried out:

1). The Nuc (*Staphylococcus aureus* nuclease) activity was measured on culture dishes containing chemically defined SA medium (Jensen and Hammer, Appl. Env. Microbiol. 59, 4363-66, 1993), which comprises a minimal amount of each of the elements required for bacterial growth, and is in particular low in zinc (10 nM of  $ZnSO_4$ ).

A solution of  $Zn^{2+}$  (20  $\mu$ l of  $ZnSO_4$  at 0.1 M) is deposited on this medium in the form of a streak crossing the dish.

After absorption of the  $Zn^{2+}$  deposit, 2 deposits of bacteria are made in the form of 2 streaks that are parallel to one another and cut across the zinc streak

such that they are perpendicular to it: a control deposit (MG1363 strain transformed with a plasmid (pVE8009) carrying the fusion Usp- $\Delta_{Sp}$ Nuc under the control of the Usp promoter) and a deposit of the MG1363 strain transformed with pVE8020 (MG1363(pVE8020)).

Overnight incubation of the dishes at 30°C allows growth of the bacteria and the creation of a gradient of decreasing  $Zn^{2+}$  concentrations by diffusion from the streak of  $ZnSO_4$ .

A staining test for nuclease activity is carried out by depositing onto the dishes a detection overlayer containing toluidine blue, and incubating at 37°C (LACHICA et al., *Appl. Microbiol.*, 21, 585-87, 1971; LE LOIR et al., *J. Bacteriol.*, 176, 5135-39, 1994): the Nuc activity is detected by the detection overlayer turning pink, forming a halo around the streaks of bacterial deposits.

While the halo observed around the streak corresponding to the control deposit is of constant size and intensity over the entire length of the bacterial streak (which indicates that neither the nuclease activity of Usp- $\Delta_{Sp}$ Nuc, nor its exportation, nor its expression, depend on  $Zn^{2+}$ ), that observed around the streak corresponding to the MG1363(pVE8020) deposit is limited to the end furthest away from the zinc deposit, where its width and its intensity are comparable to those observed for the control deposit; the intensity decreases as it gets nearer to the zinc deposit, and no halo is observed in the region of the intersection with said zinc deposit.

It therefore appears that high concentrations of zinc repress the expression of the promoter of the *zitRSQP* operon.

2) The regulation was also studied by detection of the Nuc reporter on an SDS-PAGE gel by virtue of its enzymatic activity (zymogram). For this experiment, the SA medium was again zinc-depleted, either by omitting any addition of  $ZnSO_4$  during its preparation, or by adding 0.01 mM EDTA to it. The *L. lactis* strain MG1363

(pVE8020) was inoculated into this medium, and the culture was then divided up into two portions, and 2  $\mu\text{M}$  of  $\text{Zn}^{2+}$  was added to only one of these portions. After growth at 30°C without shaking overnight, culture samples were taken, standardizing their volume according to their  $\text{OD}_{600}$  so as to obtain a number of cells equivalent to that of 1 ml of culture at  $\text{OD}_{600} = 1$ . The samples were precipitated with concentrated trichloroacetic acid, washed, lyzed in the presence lysozyme and SDS, and taken up in a loading buffer, according to the protocol described in POQUET et al. (1998, mentioned above).

The proteins were then separated according to their molecular weight on an SDS-PAGE gel containing 12.5% of acrylamide. The Nuc activity was detected as described above. Three protein forms exhibiting nuclease activity (which, according to their molecular weight, correspond to the precursor with an uncleaved signal peptide, to the mature form Nlp3- $\Delta_{\text{spNuc}}$ , and to the NucA degradation product) were detected only in the culture sample to which no addition was made. No protein having Nuc activity was detected in the sample supplemented with  $\text{Zn}^{2+}$ . These results demonstrate that the repression is complete in the presence of 2  $\mu\text{M}$  of  $\text{Zn}^{2+}$ .

#### **Effect of EDTA on the Nuc activity under the control of the $p_{\text{zn}}$ promoter**

The Nuc activity is measured on culture dishes containing M17 medium (TERZAGHI and SANDINE, *Appl. Environ. Microbiol.*, 29, 807-13, 1975) rich in zinc.

A solution of EDTA (20  $\mu\text{l}$  at 0.1 M) is deposited, as is the MG1363 strain containing the control plasmid, and the MG1363(pVE8020 strain), in the form of streaks, as described above.

After incubation at 30°C overnight, the Nuc activity is detected as described above.

The halo observed around the streak corresponding to the control deposit is, like that observed in case of the zinc, of constant size and intensity over the entire length of the streak. On the other  
5 hand, surprisingly, that observed around the streak corresponding to the MG1363(pVE8020) deposit is, in the region of the EDTA deposit, much more intense than that of the control deposit; the intensity decreases very rapidly as the distance from this intersection  
10 increases.

It therefore appears that EDTA induces expression of the promoter of the *zitRSQP* operon. The level of expression also appears to be higher than that observed at a distance from the zinc streak in the  
15 preceding experiment, and also higher than that of the control, that is controlled by the *Usp* promoter: it is therefore possible to attain a very high level of induction of  $p_{zn}$  *zitR* by means of concentrations of EDTA which do not affect the bacterial growth (such as  
20 0.1 mM in M17).

**Quantitative evaluation of the effect of EDTA on the Nuc activity under the control of the  $p_{zn}$  promoter**

In order to quantitatively evaluate the effect  
25 of EDTA on the Nuc activity expressed under the control of the promoter of the *zitRSQP* operon, the following experiments were performed:

The MG1363 strain transformed with pVE8020 is cultured in M17 medium supplemented with 5  $\mu$ g/l of  
30 erythromycin, until it reaches the exponential phase ( $OD_{600} = 0.3$ ) or the stationary phase ( $OD_{600} = 1.2$ ). This culture is divided up into 4 subcultures; EDTA is then added to 3 of them, at various final concentrations (3.3 mM; 0.33 mM; 0.033 mM); the fourth receives no  
35 addition of EDTA (0 mM EDTA). After incubation for 30 min or for 1 h 30 min, samples of each culture are taken. The number of cells of each sample is standardized by adjusting the volume so as to obtain a

number of cells equivalent to that of 1 ml of culture at  $OD_{600} = 1$ .

The cells are then lyzed and precipitated by means of treatment with concentrated (16.7%) trichloro-  
5 acetic acid, washed with acetone (80%) and taken up in 100  $\mu$ l of Tris buffer (50 mM, pH 7). 10  $\mu$ l of each sample thus treated are deposited on a dish containing medium for detecting the Nuc activity (LACHICA et al., 1971; LE LOIR et al., 1994, mentioned above). The Nuc  
10 activity is evaluated by means of the size of the halo and the intensity of the pink coloration around each deposit. For a quantitative evaluation of the level of activity, a standard range of purified Nuc protein is deposited on the same dish (4-fold serial dilution  
15 starting from 400 ng).

It is noted that the size of the halo and the intensity of the coloration varies according to the EDTA concentration which was used to treat the cells. In the absence of EDTA, a very thin halo without any  
20 clear coloration is observed; at 0.033 mM EDTA, a thin halo that is clearly colored pink is observed; at 0.33 mM, a broad halo with a very intense pink coloration is observed. No increase in the size or in the intensity of the halo is observed from 0.33 to  
25 3.3 mM. No significant difference in the size and in the intensity of the halo is noted between the addition of EDTA carried out in the exponential phase and that carried out in the stationary phase, nor between the two incubation times (30 min or 1 h 30 min).

30 These results indicate that the level of induction of the expression of  $p_{zn}$  increases with the concentration of EDTA added, up to a saturation threshold (which is reached in M17 medium, under the experimental conditions described here, for an EDTA  
35 concentration of the order of 0.33 mM, whatever the growth phase and the incubation time).

Comparison with the standard range of purified Nuc protein makes it possible to estimate that the

level of induction obtained by the addition of 0.33 mM of EDTA in M17 medium is of the order of 100.

#### **Effect of zinc on transcription of the *zit* operon**

5           The strains used are the *L. lactis* subsp *cremoris* wild-type strain MG1363, and its mutant derivative FNR (*flpA flpB* double mutant, SCOTT *et al.*, 2000, mentioned above; GOSTICK *et al.*, 1999, mentioned above). The *flp* genes are pleiotropic regulators  
10 involved in particular in zinc transport: in FNR, the intracellular zinc concentration is seven to eight times lower than that of the wild-type strain (GOSTICK *et al.*, 1999, mentioned above).

          Starting with an overnight preculture of each  
15 strain in SA medium supplemented, only for FNR, with 5 µg/µl of erythromycin and 5 µg/µl of tetracyclin, a culture is performed at 30°C in SA medium (the Zn<sup>2+</sup> concentration of which is 10 nM). In the early exponential phase (OD<sub>600</sub> 0.07 to 0.08), this culture is  
20 divided up into two parts: one (+) has ZnSO<sub>4</sub> added to it, so as to obtain a final Zn<sup>2+</sup> concentration of 2 µM (which does not affect the growth); the other (-) receives no addition. The culture is continued without modification up until the exponential phase (OD<sub>600</sub> =  
25 0.2) or stationary phase (OD<sub>600</sub> = 0.8). The bacterial RNA is then extracted according to the protocol described by RAYA *et al.*, (*J. Bacteriol.*, 180, 3174-80, 1998).

          After extraction, the RNA concentration is  
30 evaluated by measuring the OD<sub>260</sub>: 60 µg of RNA are loaded onto a 1% agarose gel. After migration and transfer onto a nylon membrane, the *zitRS* transcripts are detected by Northern blotting, with a probe specific for the *zitS* gene.

35           The results are given in figure 1.

          These results show that a specific mRNA of the size expected for *zitRS* is observed only in the absence (-) of addition of Zn<sup>2+</sup>, and never in its presence (+),



whatever the strain and whatever the growth phase at the time of the addition.

This shows 1) that the repression, by  $Zn^{2+}$ , of the expression of the *zit* operon occurs at the transcriptional level, 2) that it is complete for a  $Zn^{2+}$  concentration in the medium of 2  $\mu M$ , and 3) that it is independent of the *flp* genes, since it is exerted in the FNR mutant. The latter point indicates that the regulation by  $Zn^{2+}$  depends entirely on the *zitR* regulator.

In the absence of addition of  $Zn^{2+}$ , a very high level of expression is observed, except for the MG1363 strain in the exponential phase, where only a low expression is observed. These results indicate that, despite the very low concentration of  $Zn^{2+}$  (10 nM) in the starting SA medium, the intracellular concentration of  $Zn^{2+}$  at the time of the exponential phase when the sample was taken is still sufficient, in the case of the MG1363 strain, to strongly repress the transcription of the *zit* operon. On the other hand, in the stationary phase, after depletion of the  $Zn^{2+}$  present in the medium, the expression is very strong. In the case of the FNR strain, the 10 nM concentration of  $Zn^{2+}$  in the starting medium is insufficient to ensure, even during the exponential phase, an intracellular concentration of  $Zn^{2+}$  that represses transcription of the *zit* operon.

It therefore appears that the induction of the expression depends directly on the intracellular concentration of  $Zn^{2+}$ , and that said concentration must be very low in order to obtain maximum expression.

This can be obtained in particular:

1) By decreasing the extracellular concentration of  $Zn^{2+}$ ; this must in fact be much lower than 10 nM, where considerable repression is still observed compared with the maximum level of induction. The extracellular concentration of  $Zn^{2+}$  can, for example, be decreased by adding a chelating agent such as EDTA (whatever the growth phase), or by operating in the

stationary phase, under conditions where the  $\text{Zn}^{2+}$  initially present in the medium has been consumed by the bacteria during growth, or by a combination of these two means.

- 5           2) By using mutants in which the zinc transport is affected, and which, as a result, have a very low intracellular  $\text{Zn}^{2+}$  concentration, such as the FNR strain mentioned above.

10   **EXAMPLE 2: CONSTRUCTION OF EXPRESSION VECTORS UNDER THE CONTROL OF THE ZITRSQP OPERON REGULATORY SYSTEM**

**Construction of plasmids containing the zitRSQP operon regulatory system**

15

**Plasmid pDI11**

- The  $p_{\text{zn}}\text{-zitR}$  promoter-regulator system of the MG1363 strain is obtained by PCR amplification (DyNAzyme EXT kit from Finnzymes) of part of the
- 20  $p_{\text{zn}}\text{zitRzitS'}$  insert (GenBank U95834) of the plasmid pVE8020, with the oligonucleotides oligo 9 and oligo MUT:

Oligo 9:

5'-CTAATGAGCGGGCTTTTT-3' (SEQ ID NO: 7)

- 25 Oligo MUT:

5'-GCTCTAGAGCGGGATCCTTCATCGAACTCTTCAG-3' (SEQ ID NO: 8)

- Oligo 9 hybridizes with the multiple cloning site (MCS) of pFUN, and makes it possible to amplify any insert cloned into this vector. Oligo MUT makes it
- 30 possible to remove the potential zitS ribosome binding site (RBS) in order to facilitate the cloning of the PCR fragment: its sequence, located in the overlapping region between *zitR* and *zitS*, has two mutations (underlined) in the RBS (the wild-type sequence
- 35 5'-GGAGGAG-3' is mutated to 5'-TGAAGAG-3', complementary to 5'-CTCTTCA-3' in oligo MUT), and the two restriction sites *Bam*HI and *Xba*I (in bold).

The sequence of the region of the plasmid pVE8020 on which the amplification is performed (SEQ ID

NO: 9) is represented in figure 2. The numbers to the left of the sequence correspond to the numbering of the entire sequence of the plasmid pVE8020. The pairing regions for the primers oligo 9 and oligo MUT are represented in bold and with arrows. The sequences encoding ZitR and part of ZitS are indicated. The potential RBSs (ribosome binding sites) of *zitR* and *zitS* are boxed, and the ATG translation initiator codons are underlined. The -35 and -10 boxes of the promoter are boxed and highlighted in gray; the potential transcription initiation site is indicated by a double underlining.

The 700 bp amplification product is treated with the *Escherichia coli* DNA polymerase Klenow fragment (PolIK), and then with *Xba*I. This modified fragment is purified and cloned into the vector pFUN, digested beforehand with *Eco*RV and *Xba*I: the ligation mixture (fragment + pFUN + T4 phage ligase) is used to electroporate the *Lactococcus lactis* strain MG1363, and erythromycin-resistant clones are selected on M17 solid medium + 0.5% glucose + 5 µg/ml erythromycin. One of these clones, containing a recombinant plasmid of 8.2 kb, hereinafter called pDI11, is chosen.

The steps for constructing this plasmid are represented in figure 3a.

It contains the entire sequence encoding ZitR, and also a 5' sequence comprising the *p<sub>zn</sub>* promoter. This 5' sequence (SEQ ID NO:10) is represented hereinafter (up to the potential transcription initiation site):

GATCTGTCAGCTGGTTCAACTAGCGGTGGTCAAAGTGTAGTAATAAACTTATTGT  
TTTGATGTTTCGGCTTAAGGATGGAAGGATTTTTCAAATAAAAAAGTAAAAATAATG  
TTAACTGGTTGACATTATTTTTACTTTTGCTATATAATTAACCAGTA.

#### **Plasmid pDI12**

pDI11 is digested with *Eco*RI and *Eco*RV and treated with PolIK so as to obtain a linear fragment of 8.18 kb lacking the restriction sites of the MCS of pFUN (which makes it possible to introduce them subsequently, elsewhere in the construct), and then

treated with the T4 phage ligase. The MG1363 strain is electroporated with the ligation mixture, and an erythromycin-resistant clone containing the plasmid pDI12 is selected as described above.

5           The steps for constructing this plasmid are represented in figure 3b.

**Construction of plasmids containing a reporter gene under the control of the zitRSQP operon regulatory system**

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**Plasmid pDI24**

pDI24 comprises the following elements: a sequence encoding a reporter protein for testing the system, followed by a terminator.

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The chosen reporter protein is NucB, the form lacking a signal peptide of the Nuc nuclease from *Staphylococcus aureus* (SHORTLE, Gene, 22, 181-189, 1983). Its open reading frame is cloned into the plasmid pSEC1 (or pVE3684, CHATEL et al., Clin. Diagn. Lab. Immunol., 8, 545-551, 2001) under the control of the  $p_{nis}$  (nisin-inducible) promoter for transcription, and Usp45 signals from *L. lactis* for translation (RBSUsp45 and initiation codon) and secretion (PSUsp45 signal peptide): the entire assembly  $p_{nis}$ -RBSUsp45-PSUsp45 is cloned into pDI24.

20

25

The terminator selected is the T1T2 terminator (PESCHKE et al., J. Mol. Biol., 186, 547-555, 1985) which originates from the plasmid pVE5239 (DIEYE et al., J. Bacteriol., 183, 4157-4166, 2001).

30

To construct pDI24, pSEC1 is digested with XhoI, treated with T4 phage DNA polymerase, and digested with ClaI, so as to obtain a linear form of 3.8 kb. In parallel, pVE5239 is digested with SacI, treated with T4 phage DNA polymerase, and digested with ClaI, so as to obtain a 217 bp fragment containing the T1T2 terminator. This fragment is purified and ligated with the linear form of the vector pSEC1, and the ligation mixture is used to transform the *E. coli*

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strain TG1. Chloramphenicol-resistant clones are selected on LBT + 12.5 µg/ml chloramphenicol dishes. One of these clones, containing a 4 kb plasmid called pDI24, is selected.

5           The steps for constructing pDI24 are represented in figure 4.

#### **Plasmid pDI1224**

10           The fusion PSUsp45-NucB is placed under the control of the  $p_{zn}$ -zitR expression system in the plasmid pDI12, so as to produce the plasmid pDI1224.

15           pDI12 is digested with *Xba*I, treated with T4 phage DNA polymerase, and then digested with *Bam*HI, so as to obtain a linear form of 8.1 kb. In parallel, pDI24 is digested with *Sac*II, treated with T4 phage DNA polymerase, and digested with *Bam*HI, so as to obtain a 932 bp fragment containing the open reading frame of the NucB reporter protein (under the control of RBSUsp45 and of PSUsp45), and the transcription terminator T1T2.  
20           This 932 bp fragment is purified and ligated with the linear form of the vector pDI12, and the ligation product is used to transform MG1363. The transformants are selected on solid M17 medium + 0.5% glucose + 5 µg/ml erythromycin + 0.2 mM EDTA. The EDTA makes it  
25           possible to induce, by means of the  $p_{zn}$ -zitR system, the expression of the reporter and therefore to perform a first screening of the Nuc<sup>+</sup> phenotype of the recombinant clones. The Nuc activity assay is carried out according to the protocol described by LE LOIR et  
30           al., (J. Bacteriol. 176, 5135-5139, 1994).

          The steps for constructing pDI1224 are represented in figure 5.

35           The insert of this plasmid containing the reporter gene encoding NucB, under the control of the  $p_{zn}$ -zitR expression system, is shown diagrammatically in figure 6a.

### Plasmid pDI30

In order to quantify the level of expression controlled by  $p_{zn}$  *zitR* as a function of the environmental  $Zn^{2+}$  conditions, another reporter for cytoplasmic localization was used:  $\beta$ -galactosidase from *Leuconostoc mesenteroides* subsp. *cremoris*, encoded by the *lacLM* operon.

The *lacLM* operon was amplified by PCR from the plasmid pAMJ769 (MADSEN *et al.*, Mol. Microbiol. 32, 75-87, 1999), using the following pair of primers:

LAC5:

5'-CGCGGATCCTTTG**AAAGG**ATATTCCTC-3' (SEQ ID NO : 15)

LAC3:

5'-CCTACGTATTAGAAATGAATGTTAAAGC-3' (SEQ ID NO : 16).

The primers LAC5 and LAC3 were, respectively, designed according to the sequence of the plasmid pAK80, published by ISRAELSEN *et al.*, (Appl. Environ. Microbiol. 61, 2540-2547, 1995) and according to the sequence of the *lacLM* genes available on GeneBank under the number M92281. LAC5 overlaps the potential ribosome binding site of *lacL* (RBS, indicated in bold on the sequence), and LAC3 contains the stop codon of *lacM*. In addition, in order to make it possible to clone the PCR fragment, the *Bam*HI and *Sna*BI restriction sites were introduced at the ends of LAC5 and LAC3, respectively (underlined on the sequences). In order to construct pDI30, the recipient plasmid pDI1224 was digested with *Bam*HI and *Eco*RV so as to delete the secreted reporter PSusp45NucB, and the *lacLM* PCR product, after digestion with *Bam*HI and *Sna*BI, was inserted in its place. This ligation mixture was used to transform the *L. lactis* strain MG1363. The transformants were selected in M17 agar medium + 0.5% glucose + 5  $\mu$ g/ml erythromycin + 160  $\mu$ g/ml X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) + 0.5 mM EDTA. The addition of X-Gal and of EDTA to the medium makes it possible to screen the clones which carry pDI30 for their blue phenotype, associated with hydrolysis of the X-Gal by the  $\beta$ -galactosidase (it was verified that, in the absence of

EDTA, the clones remain white, which indicates that the medium contains sufficient  $\text{Zn}^{2+}$  to shut down the expression of LacLM).

5 The insert of this plasmid containing the *lacLM* operon encoding  $\beta$ -galactosidase, under the control of the  $p_{\text{Zn-zitR}}$  expression system, is shown diagrammatically in figure 6b.

The steps for constructing pDI30 are represented in figure 7.

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**EXAMPLE 3: QUANTIFICATION OF THE LEVEL OF INDUCTION OF A CYTOPLASMIC LOCALIZATION REPORTER GENE UNDER THE CONTROL OF THE  $P_{\text{ZnZITR}}$  PROMOTER**

15 The *L. lactis* strain MG1363 carrying the plasmid pDI30 was cultured in chemically defined SA medium, supplemented with  $1 \mu\text{M}$   $\text{Zn}^{2+}$ . Growth was effected overnight at  $30^\circ\text{C}$  without shaking.

20 The following day, the culture was diluted to approximately 1/100th in SA medium (which contains  $\text{ZnSO}_4$  at the concentration of 10 nM) and the growth was monitored by measuring the  $\text{OD}_{600}$ . At the value  $\text{OD}_{600} \sim 0.2$ , the culture was divided up into several subcultures subjected to various treatments: no addition or addition of EDTA at concentrations of 10 -  
25 30 - 50 - 100 - 300 or 500  $\mu\text{M}$ , or alternatively addition of  $\text{Zn}^{2+}$  at a concentration of  $1 \mu\text{M}$ . At various treatment times, the growth was monitored by measuring the  $\text{OD}_{600}$ , and the  $\beta$ -galactosidase ( $\beta$ -Gal) activity of the bacterial extracts was quantified by the Miller  
30 method. In the presence of ONPG,  $\beta$ -Gal produces yellow-colored O-nitrophenol which can be assayed by measuring the optical density at 420 nm; 1 Miller unit of  $\beta$ -Gal activity is defined as producing 1 nmol of O-nitrophenol per minute per unit of optical density per ml of  
35 culture.

The  $\beta$ -Gal activity measured after 1 h of treatment, as a function of the concentration of EDTA added (expressed in  $\mu\text{M}$ ), is represented in figure 8a;

the subculture supplemented with  $\text{Zn}^{2+}$  at  $1\ \mu\text{M}$  is indicated with an arrow (+  $1\ \mu\text{M}$  Zn).

In the absence of EDTA or in the presence of  $\text{Zn}^{2+}$ , a very low basal level of  $\beta$ -Gal activity is observed. In the presence of EDTA, a very clear induction of the  $\beta$ -Gal activity is observed, which depends on the concentration of EDTA: the maximum level of activity (induction by a factor  $> 100$ ) is obtained for the concentrations of  $30\ \mu\text{M}$  or  $50\ \mu\text{M}$  of EDTA, which therefore defines the optimal concentration range to be used under these conditions in order to have maximum induction.

In the course of the same experiment, the growth (curves as dotted lines) and the  $\beta$ -Gal activity (curves as solid lines) of certain subcultures were also measured as a function of time; the results are represented in figure 8b. The subcultures studied were subjected to, at  $\text{OD}_{600} \sim 0.2$  (indicated by an arrow), the following treatments: no addition ( $\square$ ); addition of  $30\ \mu\text{M}$  EDTA ( $\Delta$ ) or  $50\ \mu\text{M}$  EDTA ( $\diamond$ ); addition of  $1\ \mu\text{M}$   $\text{Zn}^{2+}$  ( $\circ$ ). At regular time intervals after these treatments, aliquots were taken in order to quantify the  $\beta$ -Gal activity of the bacterial cells. The level of induction depends both on the time of exposure to EDTA and on its concentration. The maximum induction is by a factor  $> 500$  for 3 to 4 h in the presence of  $30\ \mu\text{M}$  EDTA, which makes it possible to define the conditions for using the system.

#### **EXAMPLE 4: CONSTRUCTION OF PLASMIDS CONTAINING A SEQUENCE ENCODING A SECRETION SIGNAL PEPTIDE**

These plasmids are constructed by substitution of elements of the PSUsp45 secretion system of the plasmid pSEC with those of the Exp4 secretion system.

The sequence encoding the Exp4 signal peptide (PSExp4), accompanied by the Exp4 translation signals, i.e. its RBS (or RBSExp4) and its translation initiation codon, was amplified from the plasmid pVE8022 (POQUET *et al.*, 1998, mentioned above), using the pairs



of primers Exp4-5 + Exp4-NdeI and M13reverse + Exp4-NdeI, the sequences of which are:

M13reverse:

5'-CAGGAAACAGCTATGACC-3' (SEQ ID NO: 11);

5 Exp4-5:

5'-GTTCTAAGGATCCATTAAGGAG-3' (SEQ ID NO: 12);

Exp4-NdeI:

5'-TTTGTG**ATGCAT**TATGCAAATACAACGGCTGTTG-3' (SEQ ID NO: 13).

10 The primers Exp4-5 and Exp4-NdeI were designed based on the 5' portion of the *exp4* gene of the *L. lactis* strain MG1363 (GENBANK number U95836).

15 In Exp4-5 and Exp4-NdeI, restriction sites (in bold) are introduced at the ends of PSExp4 in order to facilitate its cloning, respectively *Bam*HI in the 5' position and *Nsi*I in the 3' position (downstream of the potential cleavage site of PSExp4). The *Nsi*I site is introduced at a position which makes it possible to clone PSExp4 in phase with NucB.

20 Moreover, Exp4-NdeI comprises an *Nde*I site (underlined) so as to allow possible cloning (of a protein of interest) in phase with PSExp4. Insertion of the *Nde*I and *Nsi*I sites introduces only two amino acids at the N-terminal end of NucB: Tyr (encoded by TAT in Exp4-NdeI) and Ala (GCA in Exp4-NdeI), which causes  
25 little disturbance of the sequence.

Amplification with Exp4-5 + Exp4-NdeI using the DNA of the plasmid pVE8022 produces a 117 bp fragment.

30 The peptide encoded by this fragment corresponds to the sequence MKKINLALLTLATLMGVSSSTAVVFA↓YA (SEQ ID NO:14) which corresponds to the sequence of the Exp4 signal peptide, up to the predicted cleavage site (indicated with an arrow) followed by the two amino acids Y and A inserted upstream of NucB.

35 After digestion with *Bam*HI and *Nsi*I, the 117 bp Exp4-5 + Exp4-NdeI fragment is inserted by ligation into pSEC1 (CHATEL et al., 2001, mentioned above) digested with the same enzymes, which allows the substitution of PSUsp45 with PSExp4.

PCR amplification with M13reverse + Exp4-NdeI using the DNA of the plasmid pVE8022 produces a 799 bp fragment. This fragment contains the Exp4 transcription, translation and secretion signals.

5       After digestion with *EcoRV* and *NsiI*, this fragment is inserted into pSEC1 digested beforehand with *XbaI*, treated with T4 phage polymerase, and digested with *NsiI*. In the resulting plasmid, the production and the secretion of NucB are thus placed  
10 under the control of the Exp4 transcription, translation and secretion signals.